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PAPER

Optimized architecture for Tyrosinase-containing Langmuir–Blodgett films to detect pyrogallol†

F. J. Pavinatto,*[ab] E. G. R. Fernandes,[ab] P. Alessio,[bc] C. J. L. Constantino,[cd] J. A. de Saja,[d] V. Zucolotto,[a] C. Apetrei,[e] O. N. Oliveira Jr[a] and M. L. Rodriguez-Mendez[b]

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The control of molecular architectures has been a key factor for the use of Langmuir–Blodgett (LB) films in biosensors, especially because biomolecules can be immobilized with preserved activity. In this paper we investigated the incorporation of tyrosinase (Tyr) in mixed Langmuir films of arachidic acid (AA) and a lutetium bisphthalocyanine ($LuPc_2$), which is confirmed by a large expansion in the surface pressure isotherm. These mixed films of AA–$LuPc_2$ + Tyr could be transferred onto ITO and Pt electrodes as indicated by FTIR and electrochemical measurements, and there was no need for crosslinking of the enzyme molecules to preserve their activity. Significantly, the activity of the immobilised Tyr was considerably higher than in previous work in the literature, which allowed Tyr-containing LB films to be used as highly sensitive voltammetric sensors to detect pyrogallol. Linear responses have been found up to 400 μM, with a detection limit of 4.87×10^{-2} μM (n = 4) and a sensitivity of 1.54 μA μM^{-1} cm^{-2}. In addition, the Hill coefficient (h = 1.27) indicates cooperation with $LuPc_2$ that also acts as a catalyst. The enhanced performance of the LB-based biosensor resulted therefore from a preserved activity of Tyr combined with the catalytic activity of $LuPc_2$, in a strategy that can be extended to other enzymes and analytes upon varying the LB film architecture.

Introduction

The quantification of polyphenols has become important for the food industry due to the health benefits, stemming from the antioxidant activity. In addition, the quality of food, beverages, oils and juices depends on phenolic compounds that are partly responsible for the self-oxidation stability and organoleptic characteristics.[1–5] Detection of phenolic compounds and derivatives is normally performed with electrochemical biosensors, especially those based on amperometry, which possess good selectivity and potential for miniaturization.[6,7] Existing applications include the assessment of polyphenols in beer,[8] tea,[9] vegetable extracts,[10,11] olive oil,[12] and wine.[13] Amperometric biosensors made with the enzyme tyrosinase (Tyr) have been used to detect phenols.[14] Tyrosinase (also referred to as polyphenol oxidase) is a dicopper enzyme[14,15] capable of both hydroxilase and oxidase activity, with which o-quinones can be generated. The o-quinones or free radical products may be electrochemically reduced at the electrode surface at very low potentials (~0V *vs.* Ag/AgCl) forming a bioelectrocatalytic amplification cycle. This prevents electrode fouling and enzyme inactivation by the polymerized products that occur at relative high potentials.[16] Thus, the bioelectrochemical current can be related to the concentration of phenols or polyphenols in the medium.

In order for an enzyme to retain its activity after immobilization onto a solid support, special strategies have to be used. For the activity of an enzyme can be strongly altered depending on the matrix used,[17] and enzyme inactivation leads to limited lifetimes in biosensors. Various methods have been used to immobilize tyrosinase for biosensors, including the incorporation of the enzyme in carbon paste,[18] glassy carbon[19] or diamond electrodes,[20] in addition to adsorption on an electrode *via* covalent bonding with glutaraldehyde,[21] or using screen printing,[22] self-assembling,[23] layer-by-layer (LbL)[24] and Langmuir–Blodgett (LB) techniques.[25,26] A particular feature of the LbL[27,28] and LB techniques is the possible adsorption of the enzyme in a lipidic layer, as well-ordered films mimicking biological membranes can preserve the enzyme structure and promote high enzymatic activity. Indeed, an enhanced activity has been observed for

[a]*Universidade de São Paulo, Instituto de Física de São Carlos, Grupo de Polímeros Bernhard Gross, São Carlos, SPBrasil. Av. Trabalhador São Carlense 400, CEP 13566-590. E-mail: pavinatto@ifsc.usp.br; Fax: +55 16 3371; Tel: +55 16 33739825*
[b]*E. T. S. Ingenieros Industriales, Universidad de Valladolid, Valladolid, Spain, CEP 47011*
[c]*Faculdade de Ciências e Tecnologia, UNESP Univ Estadual Paulista, Presidente Prudente, SP, Brasil, CEP 19060-900*
[d]*Facultad de Ciencias, Universidad de Valladolid, Valladolid, Spain, CEP 47011*
[e]*Department of Chemistry, Faculty of Sciences, "Dunarea de Jos" University of Galati, 800008 Galati, Romania*
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horseradish peroxidase in LB films of phospholipids, with the activity of the immobilized enzyme being *ca.* 23% higher than in solution.[29] A further advantage of using LbL or LB films is the control permitted by tuning parameters such as pH, ionic strength, *etc.*

One should also stress that the biosensor sensitivity can be enhanced by the introduction in the sensing films of an appropriate electron mediator, which can mediate the electron transfer between the redox enzyme and the electrode. Various molecules have been used as electron mediators, including ferrocyanide,[30] conducting polymers[31] or cobalt phthalocyanine.[32] Metallophthalocyanines (MPcs) are typical fully π-conjugated macrocyclic molecules, which may act as mediator owing to their electrochemical and catalytic properties.[33] Lanthanide bisphthalocyanines ($LnPc_2$) show a unique electrochemical behaviour dominated by the oxidation of the phthalocyanine ring.[34,35] Thin films of $LnPc_2$ have been investigated in modified-eletrodes in voltammetric sensors.[36,37] Though Tyr has been used in several sensors made with thick films or LbL films,[23,24] we were able to find only two reports of its use in LB films.[25,26] In the first paper by Cabaj and co-workers,[25] Tyr was co-spread with stearic acid and a thiophene derivative, forming a three-component Langmuir monolayer, which was then transferred as LB films. The latter films were subsequently dipped into a glutaraldehyde solution for the enzyme crosslinking with the matrix. The activity of the immobilized enzyme was only *ca.* 3.5% of the value of the free enzyme in solution,[25] probably because Tyr may have been denatured in the chloroform solution employed to produce the Langmuir film. In the second paper,[26] another strategy of immobilization was used, *i.e.* the adsorption of Tyr (initially dissolved in the subphase) onto a pre-formed Langmuir monolayer made with a mixture of linoleic acid, octadecyltrimethylammonium bromide and a conducting polymer. Even though the dissolution of Tyr in organic solvents was avoided, the activity of the immobilized enzyme was still very small, around 4% of the original activity in solution.[26] The reasons for such dramatic losses in activity are not discussed by the authors, but we may speculate that enzyme denaturing may occur upon interaction with the materials of the matrix.

In this study, we have investigated the incorporation of Tyr in mixed Langmuir films of arachidic acid (AA) and lutetium bisphtalocyanine ($LuPc_2$). Systematic experiments were performed to understand the process of enzyme adsorption from the aqueous subphase, and to obtain an efficient incorporation of Tyr at the interface. Indium tin oxide (ITO) and platinum (Pt) plates were successfully modified with the three-component LB films to produce a biosensor. For the latter, enzyme immobilization was proven, through Fourier transformed infrared (FTIR) absorption spectroscopy, and a preserved activity was confirmed with the electrochemical detection of pyrogallol. It will be shown that the activity of immobilized Tyr was 12% of its original activity in solution, and the sensing unit could detect pyrogallol with high sensitivity.

Experimental

Chemicals

Tyrosinase (EC: 1.14.18.1 from mushroom, activity of 5370 U mg^{-1}, CAS 9002-10-2), pyrogallol (FW 126.11 g mol^{-1}, 99%) and arachidic acid (Eicosanoic Acid, > 99%, TLC) were purchased from Aldrich and used as received. Lutetium bisphthalocyanine was synthesized through the direct reaction of 1,2-dicyanobenzene (phthalonitrile) with lutetium acetate according to a published procedure.[38] All other reactants were of analytical grade and used without previous purification. The deionised water used for LB films and electrochemical experiments was obtained from a Barnstead purifier, with resistivity of 18.2 MΩ cm. A phosphate buffer (0.01M, pH 7.0) was used in all experiments, and NaCl was employed to help protein adsorption, as discussed later.

Langmuir and Langmuir–Blodgett films

Langmuir and LB films were prepared in a KSV 2000 Langmuir trough (KSV Instruments, Finland) or in a NIMA model 611 trough (NIMA technologies, England), both equipped with a Wilhelmy plate to measure surface pressure. Monolayers of AA and $LuPc_2$ were prepared by spreading 100 μl of chloroform solutions with a concentration of *ca.* 1×10^{-5} M over a buffered subphase. Mixed monolayers were prepared through the mixing of the solutions in a glass flask followed by spreading. A suitable volume of each solution was chosen to get the desired molar ratio, and a variable amount (50–80 μl) of each solution was spread on the surface. All measurements were carried out at 22 ± 2 °C. Tyr was incorporated in the subphase through injection with a microsyringe just beneath the interfacial film. As will be discussed later, this was the best procedure to incorporate the enzyme in the subphase, and subsequently to the LB film. The enzyme concentration, 0.373 μg ml^{-1} (for the KSV2000 trough), was chosen to reach a 1–2% ratio between enzyme and film molecules, as this is a typical enzyme concentration to modify electrodes with the LB technique.[29] In some kinetics experiments a higher concentration was used, as will be explained. Surface pressure-molecular area (π-A) isotherms were measured by compressing the monolayer at a speed of 15 cm^2 min^{-1}.

LB films containing Tyr were deposited onto glass slides covered with ITO or Pt plates, at a surface pressure of 40 mN m^{-1}. The typical dipper speed was 3 and 2 mm min^{-1}, for the upstrokes and downstrokes, respectively. In some cases these speeds were altered by ±0.5 mm min^{-1} to optimize the monolayer transfer. ITO plates were cleaned through three successive 10 min washing steps, under sonication in acetone, chloroform and water. For the cleaning of the Pt plates the same procedure was used, but with 1 M aqueous nitric acid instead of chloroform in the second step.

FTIR measurements in the reflection mode were conducted for the AA–$LuPc_2$–Tyr LB films deposited on Pt, using a Bruker Tensor 27 spectrophotometer with a resolution of 4 cm^{-1}. For comparisons, neat LB films of AA and $LuPc_2$ were transferred onto monocrystalline zinc selenide (ZnSe) for FTIR characterization in the transmission mode. A cast film of Tyr was produced by dropping *ca.* 200 μl of a 1.68 mg ml^{-1} solution (in buffer) onto ZnSe and dried in an oven at 50 °C for 30 min. In these cases, the measurements were carried out in a Nicolet Magna IR760 spectrophotometer, also with a spectral resolution of 4 cm^{-1}.

Enzymatic activity in solution

The enzymatic activity of free and immobilized Tyr was evaluated using the spectrophotometric method, with ultraviolet-visible (UV-vis) absorption spectroscopy being conducted in a Shimadzu UV-1603 equipment. The enzyme activity was determined as U ml^{-1} of protein. One unit (U) of enzyme was defined as the amount of enzyme that causes an increase in absorbance of 0.001 per min at 25 °C.[39] For the free enzyme, 5.0 μl of a standard solution of Tyr (1.68 mg ml^{-1}) were added to 2.5 ml of a 13 mM pyrogallol solution prepared in the phosphate buffer at pH 7.0. The changes in the absorption band at 420 nm (associated with the presence of o-quinone products) were recorded during 2 min at 5 s intervals. For the immobilized enzyme, the electrode (ITO covered with the LB film) was inserted in a buffer containing 13 mM pyrogallol, and the absorbance at 420 nm was monitored with time.

The kinetics parameters, *i.e.* the maximum reaction rate (V_{max}) and the Henri–Michaelis–Menten constant (K_m), were calculated for the enzyme in solution using the Lineweaver–Burk method.[40] This was performed by measuring the enzymatic activity at 420 nm using pyrogallol concentrations ranging from 0.1 to 10 mM.

Electrochemical measurements: detection of pyrogallol

Electrochemical measurements were carried out in an EG&G PARSTAT 2273 potentiostat/galvanostat using a conventional three-electrode cell. The conducting substrates covered with LB films were used as working electrodes, and the reference and counter electrodes were Ag|AgCl/KCl 3M l^{-1} and a Pt plate (1.5 cm^2), respectively. Cyclic voltammograms were obtained from −0.50 to +0.50 V at a scan rate of 0.10 or 0.05 V s^{-1}. All experiments were carried out at a controlled temperature of 25.0 ± 0.2 °C, and the pyrogallol solution was prepared in a phosphate buffer 0.01 M, pH 7.0.

Results and discussion

Langmuir films

Properties of the AA–$LuPc_2$ matrix. The fabrication of neat LB films of $LuPc_2$ was described for the first time in 1989 by Liu and co-workers,[41] and since then these films have been used in sensing units[42] and had their electrochemical properties studied in detail.[43,44] Mixed LB films of a $LuPc_2$ derivative and AA were also analyzed with Brewster Angle Microscopy (BAM), where the incorporation of AA made the phthalocyanine monolayers more homogeneous. This is advantageous for the building of stable, reproducible recognition elements in sensors.[45] Indeed, AA assists the transfer of good-quality LB films, which is the reason why mixed $LuPc_2$–AA LB films were chosen here as matrix for Tyr immobilization.

The interaction between AA and $LuPc_2$ at the interface was investigated by analysing the π-A isotherms shown in Fig. 1. The curves were obtained over a buffered subphase, which was required to preserve Tyr structure and activity. The π-A isotherm of AA (black curve) on the buffer is slightly different from that for a pure water subphase.[45] The liquid-to-solid transition is extinguished, and the extrapolated area (the area value at which a tangent line to the steeper part of the isotherm crosses the *x*-axis) is increased from *ca.* 20 $Å^2$ mol^{-1} to *ca.* 27 $Å^2$ mol^{-1}. The latter reflects a decrease in the packing density of the film, probably induced by an increased repulsion between the hydrophilic headgroups in the presence of the buffer. The π-A isotherm for $LuPc_2$ on a buffered subphase also resembles that on pure water,[46] except for the appearance of a less compressible phase at small pressures. The extrapolated area measured from the curve in Fig. 1 is *ca.* 87 $Å^2$ mol^{-1}, to be compared with ~80 $Å^2$ mol^{-1} reported in the literature.[46] This indicates that the $LuPc_2$ molecule adopts the same orientation, relative to the surface, as in films formed on water.

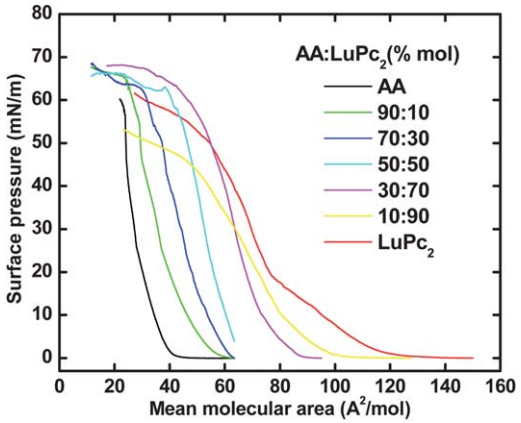

Fig. 1 π-A isotherms for mixed AA–$LuPc_2$ monolayers on the buffer. The molar proportion is given in the inset.

The curves for the mixed films lie in between those for the pure monolayers, and perhaps the only noteworthy feature is the lower collapse pressure for the film with 90% in mol of $LuPc_2$. The possible molecular-level interaction between the components was tested by comparing the measured area per molecule and the calculated area A_{12}, using the additive rule:[47] $A_{12} = X_1A_1 + X_2A_2$ (where X_n and A_n are, respectively, the molar fraction and the area occupied in a pure monolayer of each material). If the areas are the same, then the two compounds have no molecular-level interactions. Fig. 2 shows for the pressure of 20 mN m^{-1} that the measured area is slightly higher than the calculated one. The

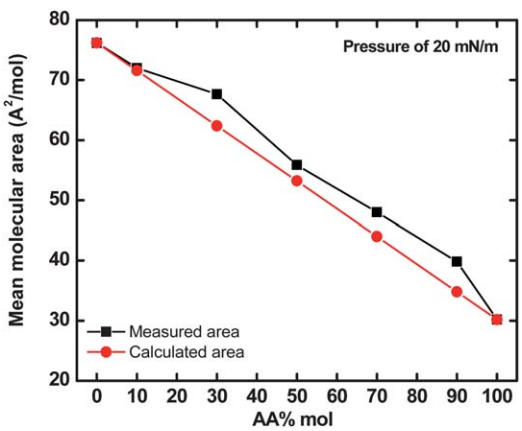

Fig. 2 Measured (black squares) and calculated (red circles) areas for the mixed AA–$LuPc_2$ monolayers *versus* molar proportion of AA.

small, positive deviation reflects some excess of free energy for the mixed monolayers at this state of packing, but the maximum difference is *ca.* 8%. This is rather small compared to mixed systems where there is a strong interaction.[48]

BAM images reported previously show that these mixed monolayers are more homogeneous than the pure phthalocyanine film.[45] This explains why the deviation from the ideal in Fig. 2 is so small, being consistent with the finding that the incorporation of AA makes the Langmuir film of phthalocyanine more homogeneous. The increased miscibility is advantageous for preparing biosensors as the electron mediator ($LuPc_2$) is distributed homogeneously in the sensing layer. A mixed film with an intermediate quantity of phthalocyanine (AA–$LuPc_2$ 1 : 1) was used in further experiments of Tyr immobilization, because this composition was suitable for electrochemical measurements. The studies of enzyme incorporation to the matrix are discussed in the next section.

Incorporation of Tyrosinase to the AA–$LuPc_2$ matrix. The first step in studying incorporation of water-soluble biomolecules into Langmuir monolayers is to select the strategy to add the guest molecules, as the results may differ.[49,50] Perhaps the most reliable, reproducible method is the protein injection at different points of the subphase just beneath the Langmuir film,[50,51] which is the procedure adopted in this work.

Before incorporating Tyr to the mixed matrix of AA–$LuPc_2$, the intrinsic surface activity of the protein was evaluated through measurements of surface pressure. For a bare air–water interface, no change in the surface pressure of a Tyr solution (0.373 μg ml^{-1}) in a phosphate buffer (0.01 M) was detected during 130 min. In addition, even after sweeping the interface with the movable barriers of the Langmuir through, the surface pressure remained null, demonstrating the absence of an interfacial film. This was also observed for solutions with increased concentrations by 3 or 5 fold, pointing to the lack of intrinsic surface activity of Tyr. Even when 0.10 M of NaCl was added to the protein solution in order to favor protein migration to the interface owing to the salting-out effect,[52] there was no increase in pressure after 130 min. However, after sweeping the interface with the barriers, an isotherm could be obtained with surface pressures up to 19 mN m^{-1}, characteristic of a Gibbs monolayer, as shown in Fig. 3. In subsidiary experiments (results not shown) we noted that these films on a buffered subphase containing salt were stable for up to 2 h. Also, we tried to deposit these films in a LB fashion and use them in biosensing. Unfortunately, these pure Tyr LB films detached easily from the substrate when immersed in the electrochemical medium, and the peak current was very small to allow for the detection. This emphasizes the importance of the matrix used.

When a mixed 1 : 1 Langmuir film of AA–$LuPc_2$ was at the air/water interface, Tyr adsorbed even for the non-compressed film (initial surface pressure of *ca.* 0.5 mN m^{-1}), which was recorded in terms of a change in surface pressure ($\Delta\pi$). The kinetics curve in Fig. 4 comprises three steps: first, fast increase in π up to 1.80 mN m^{-1} followed by a small drop of *ca.* 0.25 mN m^{-1}, and a subsequent slower increase. The total $\Delta\pi$ of 1.75 mN m^{-1} was measured after 90 min. Approximately 83% of this increase was attained within the first 20 min of the experiment. It is then inferred that 20 min is a sufficiently long waiting time for Tyr adsorption, and this was the waiting time used prior to the LB depositions.

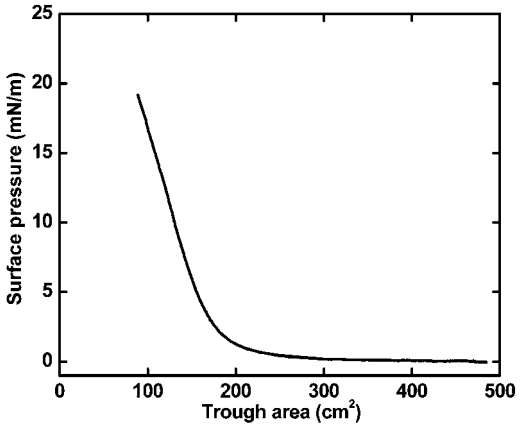

Fig. 3 Surface pressure-area isotherm for an interfacial tyrosinase layer obtained with adsorption of the enzyme initially dissolved in the buffered subphase at the concentration of 0.373 μg ml^{-1}. The *x*-axis is given in units of trough area because the amount of material at the interface is unknown.

At the end of the measurements, to obtain the kinetics shown in Fig. 4, the interface was swept with the movable barriers and the π-A isotherm was recorded. The curve obtained is shown in Fig. 5 (red curve) together with the isotherm for the same monolayer formed over pure buffer (black curve). The incorporation of Tyr to the mixed monolayer of AA and $LuPc_2$ is confirmed by the expansion of the isotherm, which is shifted to higher values of mean molecular area. Such expansion is reduced with increasing surface pressure, resulting in the coincidence of both curves at the collapse pressure. This suggests that Tyr can no longer be stable in the film, being rather expelled from the interface at high packing density. It should be noted, however, that Tyr could still interact with the monolayer if located on a sub-surface, as it has been reported for chitosan (a biopolymer) and Langmuir films of phospholipids.[53]

The mixed AA–$LuPc_2$ monolayer onto which Tyr was adsorbed is stable up to high pressures (30–40 mN m^{-1}), which then

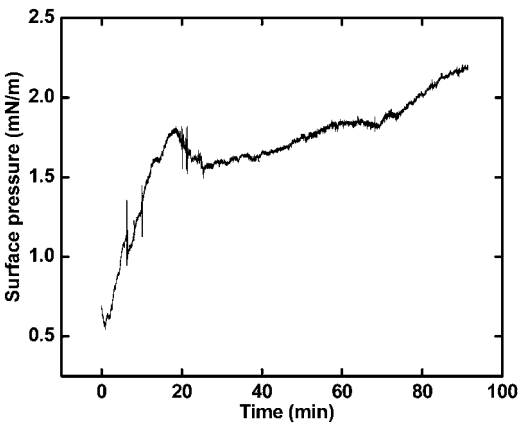

Fig. 4 The kinetics of adsorption for tyrosinase initially dissolved in the buffered subphase (0.373 μg ml^{-1}), onto a mixed Langmuir monolayer of AA–$LuPc_2$ (1 : 1 mol) at an initial π of 0.5 mN m^{-1}.

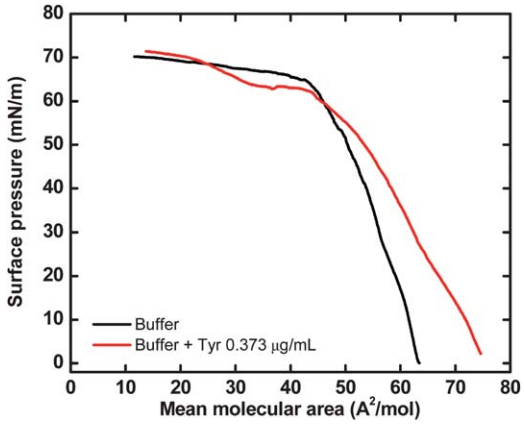

Fig. 5 π-A isotherms for a mixed AA–$LuPc_2$ (1 : 1 mol) monolayer formed on pure buffer (black curve), and on a buffered subphase containing tyrosinase in the concentration of 0.373 µg ml^{-1} (red curve).

allows for the deposition of LB films to modify electrodes, as reported in the next section.

Langmuir–Blodgett films: modified-electrodes. Pt and ITO electrodes were modified with LB films containing Tyr. An area of approximately 1 cm^2 of each face of the substrates was covered for all LB films. The successful transfer was firstly confirmed by estimating the transfer ratio (TR = reduced area of the Langmuir film/surface area of the substrate immersed). For Pt substrates TR was always higher than one for the upstrokes, mostly around 1.5, and it was close to zero for the downstrokes, which suggests the formation of a Z-type LB film. For ITO substrates, TR was positive (*ca.* 1.5) for the upstrokes, but negative (around −0.5) for the downstrokes, pointing to some material desorption from the substrate and re-spreading onto the surface. Despite these differences in TR, both films (onto ITO and Pt) were macroscopically identical in terms of homogeneity and substrate coverage with no region of the substrate appearing uncovered in a visual inspection. Films with up to 21 layers were deposited.

FTIR measurements were conducted to confirm the transfer of Tyr to the LB films. Fig. 6 shows the spectrum for an 11-layer LB film of AA–$LuPc_2$ + Tyr onto Pt. Also, the spectra for 21-layer LB films of AA and $LuPc_2$, and for a cast film of Tyr (all deposited on ZnSe) are shown for comparison. The assignments for the main peaks in the neat films are given in Table 1. The spectrum for the AA-$LuPc_2$ + Tyr LB film shows the characteristic peaks of all three components. In particular, the amide I and amide II bands of the enzyme, respectively at 1650 and 1540 cm^{-1}, are observed to confirm that Tyr was transferred onto the solid support along with the matrix material.

The proposed structure for the modified electrode is shown in Fig. 7, according to which most enzyme molecules are believed to be incorporated to the matrix as a (mono)layer just beneath the hydrophilic layer containing the carboxylic heads of AA. However, a minor portion of the total enzyme is probably inserted into the matrix, as suggested by the small expansion observed in the isotherm in Fig. 5 at $\pi = 40$ mN m^{-1} (the deposition pressure). These modified electrodes were further characterized regarding the activity of the immobilized enzyme and were used in the electrochemical detection of pyrogallol.

Enzymatic activity of the LB films of AA:$LuPc_2$:Tyr. The incorporation of Tyr in the LB films could be further confirmed by studying the enzymatic activity of the films immersed in

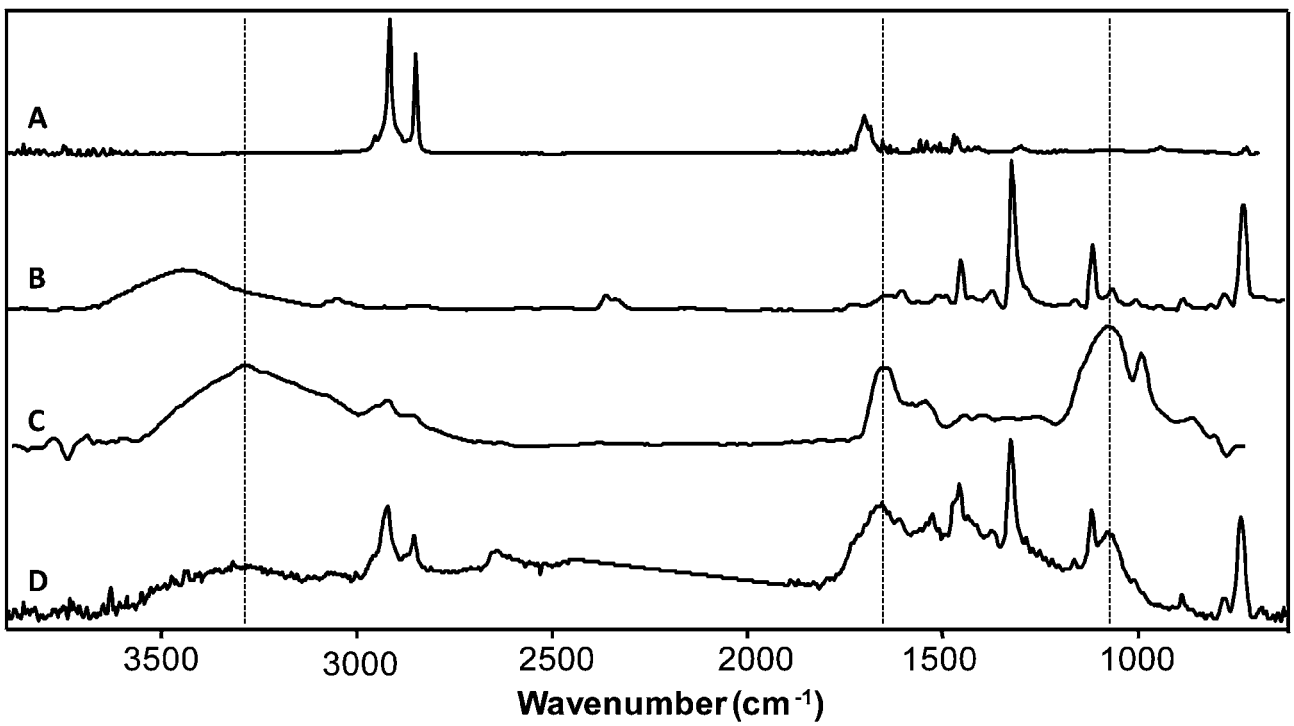

Fig. 6 FTIR spectra for: [A] 21-layer LB film of AA on ZnSe; [B] 21-layer LB film of $LuPc_2$ on ZnSe; [C] cast film of Tyrosinase on ZnSe; [D] 11-layer LB film of AA–$LuPc_2$ + Tyr on Pt.

Table 1 Assignments for the FTIR spectra of the pure LB films in Fig. 6

Film Material	Peak position (cm−1)	Assignment	Reference
$LuPc_2$	727 (s)	CH wagging	54
	1116 (s)	CH bending	
	1322 (s)	Isoindole stretching	
	1452 (s)		
Arachidic Acid	1701 (s)	C = O stretching	55
	2849 (s)	CH_2 symmetric stretching	
	2917 (s)	CH_2 antisymmetric stretching	
	2930 (w)	CH_3 symmetric stretching	
Tyrosinase	1073 (bp)	Amide III - complex combination of vibrations	56–59
	1540 (bp)	Amide II - NH bending + CN stretching	
	1650 (bp)	Amide I - C = O stretching + CN stretching	
	3000–3500 (bb)	H-bonded NH and OH vibrations combined	

[a] s - strong peak; w - weak peak; bp - broad peak; bb - broad band.

a pyrogallol solution using UV-vis spectroscopy. The results were compared with the enzymatic activity of free Tyr added to a pyrogallol solution. Pyrogallol can suffer a self -oxidation process yielding oxygen reactive species (ORE), and then leading to the formation of o-quinone, purpurogallin and other products.[60] The enzymatic catalysis results in a colour change to light yellow assigned to the band at 420 nm with the formation of purpurogallin.[61,62] An enzyme unit (U) can be defined as the enzyme quantity able to convert 1 μmol of pyrrogallol per minute at pH 7.0 and 25 °C.

The UV-vis absorption spectra of a pyrogallol solution in the buffer are characterised by two bands at 220 nm and 266 nm, and a shoulder close to 240 nm. The solution of the enzyme Tyr shows characteristic bands at 280 nm (transitions n–π*), which is common to a variety of enzymes owing to the presence of aromatic aminoacids. A second intense band at 200 nm with a shoulder at 220 nm (π–π* transition) is also observed (ESI, †). Upon addition of Tyr to the pyrogallol solution, two new bands at 315 and 420 nm appear owing to the purpurogallin. The intensity of these bands increases with time (at high concentration of pyrogallol the band at 315 nm also appears due to the self-oxidation process). The same behaviour was observed with the immersion of an as-deposited LB film containing AA + Tyr (10 layers) in a solution of pyrogallol at 13 mM. Changes in the UV-vis spectra were monitored with time (ESI, †). Upon interaction between the film and the pyrogallol solution, two new bands were formed at 370 nm and 420 nm, which correspond to the formation of the intermediate compound purpurogallin.[62] The intensity of these bands also increased with time (ESI, †).

The enzyme activity for free Tyr was 178 U ml^{-1}, while the immobilized Tyr had 21 U ml^{-1} (*ca.* 12% of recovery). Though this activity is much smaller than in free solution, it is considerably higher than the values quoted in the literature for Tyr in LB films. Significantly, Cabaj *et al.*[25] obtained 3.5% for Tyr covalently immobilized with glutaraldehyde on LB films. This low enzyme activity may be due to denaturing because Tyr was dissolved in chloroform. When Tyr was adsorbed from the subphase, as we did in the present work, Cabaj *et al.*[26] obtained 4%. This is again lower than our results, possibly because of steric hindrance toward the analyte owing to the cross-linking of the proteins.

The kinetics parameters V_{max} and K_m for Tyr in solution were evaluated. V_{max} describes the rate of reaction between Tyr and its substrate, while K_m defines the enzyme affinity to the substrate, the lower the K_m the higher the affinity toward the substrate. From the kinetics studies in free solution, V_{max} and K_m were 0.278 μM min^{-1} and 0.78 mM, respectively. Such value of K_m is comparable to that obtained for Tyr immobilized in the LB film, as will be discussed in the next section.

Electrochemical characterization and biosensing properties. The cyclic voltammetry (CV) diagrams of 20-layer LB films of neat AA and AA + Tyr are shown in Fig. 8. The CV for the AA + Tyr electrode in the buffer shows a low background current without a detectable signal. Upon addition of pyrogallol to the buffer solution, a redox process appeared in the CV with the shape of catalytic wave at $E_{1/2} = -0.20$ V (Fig. 8). This result is in good agreement with those for other matrices.[63]

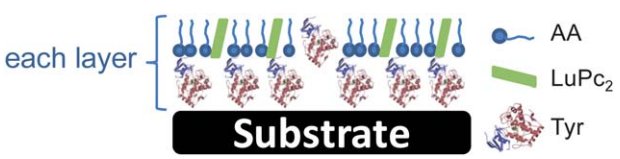

Fig. 7 Idealized structured for the modified electrodes covered with the mixed AA–$LuPc_2$ + Tyrosinase LB multilayers.

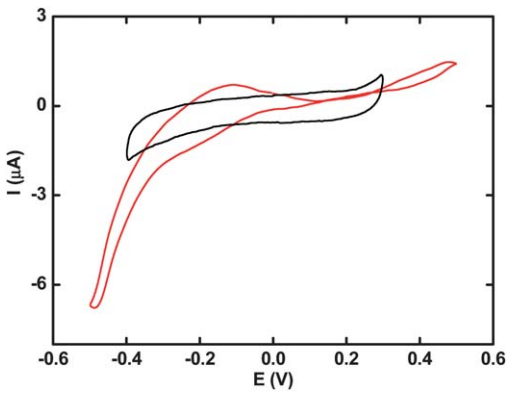

Fig. 8 Cyclic voltammograms of an ITO electrode covered with a 20-layer LB film of AA (black curve) and AA + Tyr (red curve) immersed in a 1×10^{-4} M pyrogallol solution with a scan rate of 0.10 V s^{-1}.

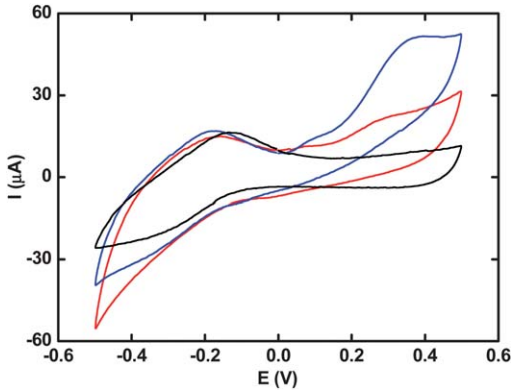

Fig. 9 Cyclic voltammograms for ITO electrodes covered with 20-layer LB films: [black curve] = AA–$LuPc_2$ LB film immersed in buffer; [red curve] = AA–$LuPc_2$ LB film immersed in a pyrogallol 1×10^{-4} M; [blue curve] AA–$LuPc_2$ + Tyr LB film immersed in pyrogallol 1×10^{-4} M.

The voltammograms with 20-layer LB films of AA–$LuPc_2$ immersed in buffer or in a pyrogallol solution, and the voltammogram for a 20-layer LB film of AA–$LuPc_2$ + Tyr, in pyrogallol solution, are shown in Fig. 9. For the film without enzyme immersed in the buffer, a cathodic peak appeared which corresponds to the reduction of the neutral form of the phthalocyanine molecule $[Pc_2–Ln_3 + Pc_2^-]^- \leftrightarrow [Pc_2–Ln_3 + Pc_2\text{-}]^0$ at E = −0.20 V.[64,65] When this film is immersed in pyrogallol (1×10^{-4} M), the CV shows the redox response of phthalocyanine at −0.20 V. The electrochemical activity of pyrogallol is also observed, characterized by the expected redox pair at $E_{1/2} = 0.15$ V, similar to the peak using Tyr at a slightly higher potential. In addition, a new intense anodic peak at 0.40 V appears. This effect could be associated with the electrochemical oxidation of pyrogallol that is catalysed by an enzymatic-like activity of the bisphthalocyanine, which has already been described for other phthalocyanines.[66,67]

The response of the mixed LB films containing AA–$LuPc_2$ + Tyr was tested in buffer in the range from −0.50 V to +0.50 V at a scan rate of 0.10 V s^{-1}. The mediator effect of the bisphthalocyanines was confirmed by comparing the response of the LB films of AA containing Tyr with and without the mediator ($LuPc_2$). The signals associated with pyrogallol can be observed in both cases. However, the mediator increases the signals by *ca.* 20 times.

The kinetics of the electrode response was investigated by recording the CV at scan rates ranging from 0.01 to 0.20 V s^{-1}. Fig. 10A shows an increased peak intensity with the scan rate, while the plots of peak intensity at 0.20 V and 0.40 V *versus* the square root of the scan rate are shown in Fig. 10B and 10C, respectively. The Randles–Sevcik equation is obeyed, thus indicating that the enzymatic reactions are diffusion-controlled processes.

The sensing properties of the LB films were evaluated through the electrochemical response toward pyrogallol solutions ranging from 0.2×10^{-4} to 4.0×10^{-4} M. As shown in Fig. 11, the peak intensity at 0.40 V and 0.15 V increased with pyrogallol concentration, confirming the assignment of these peaks to the electroactivity of pyrogallol. The response is highly reproducible with a standard deviation (n = 4) of *ca.* 2%.

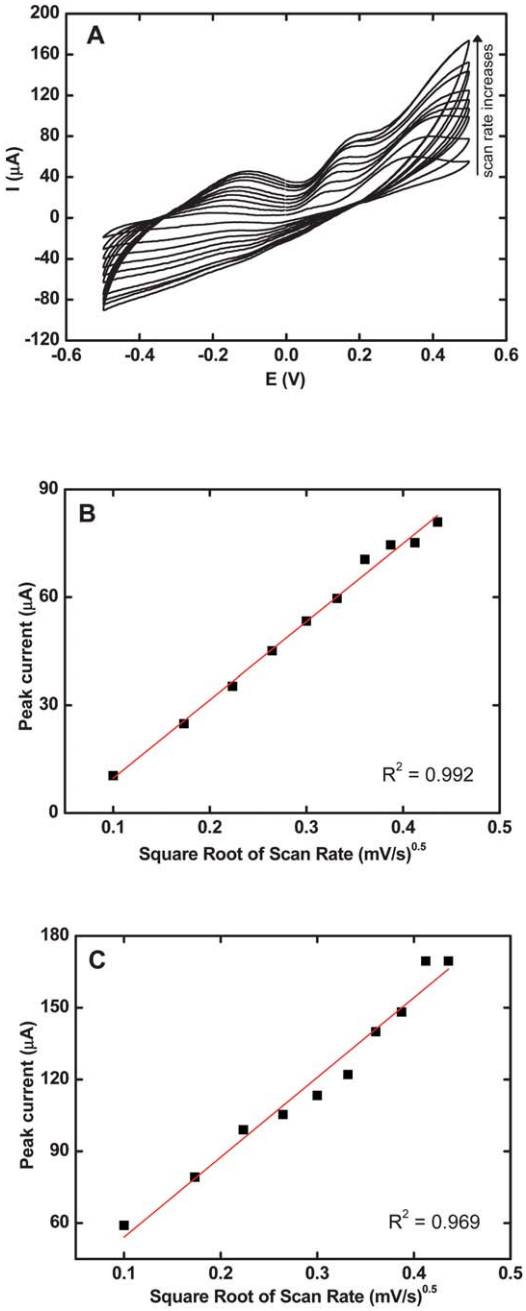

Fig. 10 [A] Cyclic voltammograms for a 20-layer AA–$LuPc_2$ + Tyr LB film (ITO) immersed in a pyrogallol solution, obtained at different scan rates ranging from 0.01 to 0.20 V s^{-1}. [B] Peak maximum at 0.20 V *versus* the square root of the scan rate; [C] The same for the peak at 0.40 V.

A calibration curve shown in Fig. 12 was obtained with the response from the LB film-based biosensor at 0.40 V using Pt as substrate. The electrochemical wave was essentially the same as for an ITO electrode, but the electrochemical response increased significantly. A linear response up to 400 μM ($R^2 = 0.9932$) with a sensitivity of 1.54 μA μM^{-1} cm^{-2} was achieved for pyrogallol. Previous papers reported a dynamic range < 100 μM for phenols.[68] The limit of detection (LD) was 4.87×10^{-2} μM (n = 4), calculated according to the $3\sigma_0$ m^{-1} criteria,[8] with m being the slope of the calibration curve and σ_0 being the standard deviation

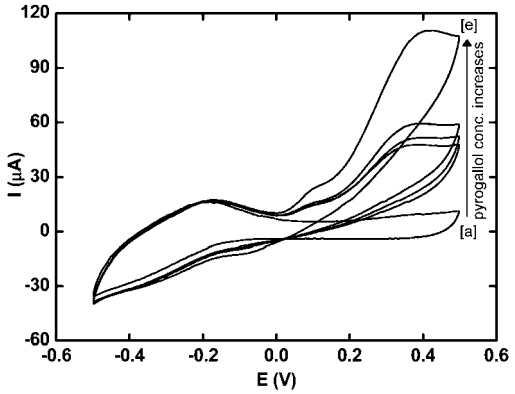

Fig. 11 Cyclic voltammograms for a 20-layer AA–$LuPc_2$ + Tyr LB film (ITO) immersed in pyrogallol solutions with different concentrations: [a] pure buffer; [b] 1×10^{-4} M; [c] 2×10^{-4} M; [d] 3×10^{-4} M; and [e] 4×10^{-4} M. Scan rate 0.05 V s^{-1}.

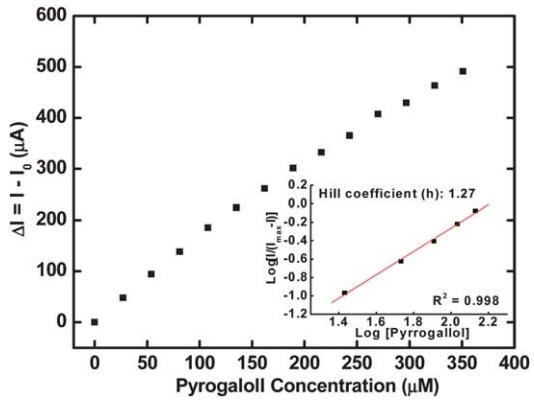

Fig. 12 Calibration curve for a 21-layer LB film of AA–$LuPc_2$ + Tyr deposited onto Pt, obtained for pyrogallol at V = 0.40 V. The inset represents the Hill plot (h = 1.27, $R^2 = 0.998$).

of the blank signal (pure buffer). The Hill coefficient was calculated by applying the Hill equation.[68,69] This coefficient is used in determining the degree of cooperativeness of the ligand binding to the enzyme or active site. A h > 1 indicates positively cooperative reaction (since one ligand molecule is bound to the enzyme, its affinity to other ligand molecules increases). A value h < 1 indicates a negative cooperactivity and h = 1 represents a Henri–Michaelis–Menten kinetics. For the LB based biosensor, the Hill coefficient (with $R = 0.998$) was 1.27, as shown in the inset of Fig. 12. The h > 1 can be explained as $LuPc_2$ may act as a catalyst, since it is capable to catalyze pyrogallol oxidation.

The apparent Michaelis–Menten kinetics parameter, K_m^{app}, can be determined from the calibration curve using the Lineweaver–Burk plot.[40] For the 21-layer LB film of AA–$LuPc_2$ + Tyr, Fig. 13 yields $K_m^{app} = 1.31$ mM. This value is close to that measured for the free enzyme (0.78 mM), which means that Tyr retains a relatively high activity after immobilization.

In an earlier work[70] we demonstrated that Tyr-containing LB films could also be used to determine low concentrations of various antioxidants, in addition to the pyrogallol studied here. The sensitivity, however, was not as high as in the present work, as the limit of detection was two orders of magnitude higher. This

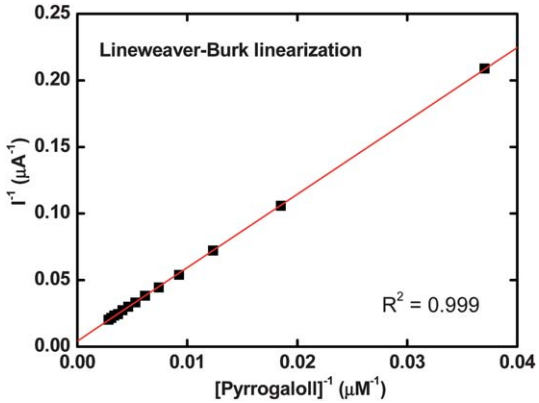

Fig. 13 Calculation of the K_m^{app} from the Lineweaver–Burk plot derived from the calibration curve for the 21-layer LB film of AA–$LuPc_2$ + Tyr biosensor (Fig. 12). $R^2 = 0.999$.

is probably because a crosslinking step with glutaraldehyde was used for the Tyr-containing LB film in ref. 70. It appears, therefore, that optimizing the film-fabrication conditions, so as to avoid crosslinking, may be advantageous for reaching high sensitivities.

Conclusions

In this study we have produced nanostructured Langmuir–Blodgett (LB) films containing the enzyme tyrosinase (Tyr). The enzyme was first incorporated to mixed Langmuir films of arachidic acid (AA), employed as matrix, and lutetium bisphtalocyanine ($LuPc_2$), which served as electron mediator in the electrochemical analysis. Despite the water-soluble character of Tyr, it could migrate to the interface with the addition of NaCl into the subphase, thus forming a Gibbs monolayer. The incorporation to the mixed (1 : 1 in mol) AA:$LuPc_2$ film was confirmed by a $\Delta\pi$ of *ca.* 2 mN m^{-1} for an expanded monolayer, and with changes in the surface pressure (π-A) isotherm of the mixed Langmuir films.

Pt and ITO electrodes were modified with Z-type Tyr-containing LB films. Such films were tested for the biosensing of the antioxidant pyrogallol. The activity of the immobilised Tyr was 12% of its original activity in solution, which was much larger than the values quoted in the literature for LB films. No significant decrease in response was observed after at least 10 measurements with good reproducibility and standard deviation (n = 4) of *ca.* 2%. The response of the biosensor was linear up to 400 μM for pyrrogallol (at an applied potention of 0.40 V), with a sensitivity of 1.54 μA μM^{-1} cm^{-2} and limit of detection of 4.87×10^{-2} μM (n = 4). A Hill coefficient above one confirmed that $LuPc_2$ acts as a catalyst for pyrogallol.

The combined action of Tyr and $LuPc_2$ yield high-performance sensors, highlighting the opportunities for fabricating LB films with varied architectures in order to seek synergy in the interaction between the film components.

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